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Date of Filing	:	02 JULY 1999

Application number	:	9903146-0

Applicants	:	INSTITUTE OF MOLECULAR AGROBIOLOGY

Title of Invention	:	GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES AS TARGETS FOR ENGINEERING DISEASE RESISTANCE

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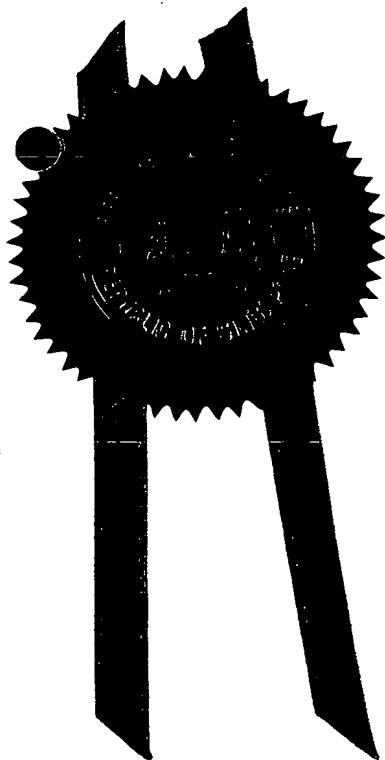
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08 December 1999

9903146-0

PATENTS FORM 1

SINGAPORE
THE PATENTS ACT
(CHAPTER 221)
THE PATENTS RULES

The Registrar of Patents
Registry of Patents

REQUEST FOR THE GRANT OF A PATENT

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION.

I Title of Invention	GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES AS TARGETS FOR ENGINEERING DISEASE RESISTANCE			
II Applicant(s) *(See note 2)*	(a) Name	Institute of Molecular Agrobiology		
	Body Description/ Residency	Incorporated under the laws of Singapore		
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	City			
	State	117604		
	Country	Singapore		
	(b) Name			
	Body Description/ Residency			
	Street Name & Number			
	City			
	State			
	Country			
	(c) Name			
	Body Description/ Residency			
	Street Name & Number			
	City			
	State			
	Country			
III Declaration of priority *(See note 3)*	Country/Country Designated		File No	
	Filing Date			
	Country/Country Designated		File No	
	Filing Date			
	Country/Country Designated		File No	
	Filing Date			

Patents Form 1 - 1

IV	Inventors *(See note 4)* (a) The applicant(s) is/are the sole/joint inventor(s)			

(a) The applicant(s) is/are the sole/joint inventor(s):

	☐ Yes		X No
	X Yes		☐ No

(b) A statement on Patents Form 8 is/will be furnished

V	Name of Agent (if any) *(See note 5)*	Ella Cheong & G Mirandah		

VI	Address for Service *(See note 6)*	Block/Hse No		Level No	
		Unit No/PO Box 0931		Postal Code	911732
		Street Name			
		Building Name Raffles City			

VII	Claiming an earlier filing date under section 20(3), 26(6) or 47(4) *(See note 7)*	Application No				
		Filing Date				

VIII	Invention has been displayed at an International Exhibition *(See note 8)*	☐ Yes X No

IX	Section 114 requirements *(See note 9)*	The invention relates to and/or used a micro-organism deposited for the purposes of disclosure in accordance with section 114 with a depository authority under the Budapest Treaty. ☐ Yes X No

X.	Check List *(To be filled in by applicant or agent)*	A. The application contains the following number of sheet(s):-		
		1 Request	3	sheets
		2 Description	31	sheets
		3 Claim(s)	4	sheets
		4 Drawing(s)	8	sheets
		5 Abstract	1	sheets

B.	The application as filed is accompanied by:-		
1	Priority document		
2	Translation of priority document		
3	Statement of Inventorship & right to grant		
4	International Exhibition Certificate		

X.	Signature(s) *(See note 10)*	Applicant (a)	
		Date 2 July 1999	
		Applicant (b)	
		Date	
		Applicant (c)	
		Date	

Patents Form 1 - 2

SECOND SCHEDULE - <u>continued</u>

NOTES:

1. This form when completed, should be brought or sent to the Registry of Patents together with the prescribed fee and 3 copies of the description of the invention, and of any drawings.

2. Enter the <u>name and address of each applicant</u> in the spaces provided at paragraph II. <u>Names of individuals</u> should be indicated in full and the surname or family name should be underlined. <u>The names of all partners</u> in a firm must be given in full. The <u>place of residence of each individual</u> should also be furnished in the space provided. Bodies corporate should be designated by their <u>corporate name</u> and <u>country of incorporation</u> and, where appropriate, the <u>state of incorporation</u> within that country should be entered where provided. Where more than three applicants are to be named, the names and address of the fourth and any further applicants should be given on a <u>separate sheet</u> attached to this Form together with the <u>signature of each of these further applicants</u>.

3. The declaration of priority at paragraph III should state the date of the previous filing, the country in which it was made, and indicate the file number, if available. Where the application relied upon in an International Application or a regional patent application e.g. European patent application, one of the countries designated in that application [being one falling under the Patents (Convention Countries) Order] should be identified and the name of that country should be entered in the space provided.

4. Where the applicant or applicants is/are the sole inventor or the joint inventors, paragraph IV should be completed by marking the "YES" Box in the declaration (a) and the "NO" Box in the alternative statement (b). Where this is not the case, the "NO" Box in declaration (a) should be marked and a statement will be required to be filed on Patents Form 8.

5. If the applicant has appointed an agent to act on his behalf, the agent's name should be indicated in the spaces available at paragraph V.

6. An address for service in Singapore to which all documents may be sent must be stated at paragraph VI. It is recommended that a telephone number be provided if an agent is not appointed.

7. When an application is made by virtue of section 20(3), 26(6) or 47(4), the appropriate section should be identified at paragraph VII and the number of the earlier application or any patent granted thereon identified.

8. Where the applicant wishes an earlier disclosure of the invention by him at an International Exhibition to be disregarded in accordance with section 14(4)(c), then the "YES" Box at paragraph VIII should be marked. Otherwise the "NO" Box should be marked.

9. Where in disclosing the invention the application refers to one or more micro-organisms deposited with a depositary authority under the Budapest Treaty, then the "YES" Box at paragraph IX should be marked. Otherwise the "NO" Box should be marked.

10. Attention is drawn to rules 90 and 105 of the Patent Rules. Where there are more than three applicants, see also Note 2 above.

11. Applicants resident in Singapore are reminded that if the Registry of Patents considers that an application contains information the publication of which might be prejudicial to the defence of Singapore or the safety of the public, it may prohibit or restrict its publication or communication. Any person resident in Singapore and wishing to apply for patent protection in other countries must first obtain permission from the Singapore Registry of Patents unless they have already applied for a patent for the same invention in Singapore. In the latter case, no application should be made overseas until at least two months after the application has been filed in Singapore.

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Application Filing Date :   /   /

Request received on   :   /   /

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TITLE OF THE INVENTION
GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES
AS TARGETS FOR ENGINEERING DISEASE RESISTANCE

CROSS-REFERENCES TO RELATED APPLICATIONS
Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT
5

Not applicable.

BACKGROUND OF THE INVENTION

1.   Field of the Invention

10   The present invention relates to global regulators of bacterial pathogenic genes, and their use to confer disease resistance.

2.   Description of the Related Art
        A bibliography follows at the end of the Detailed Description of the Invention. The listed references
15   are all incorporated herein by reference.
        Cell-to-cell communication via small signal molecules is not only of vital importance to multi-celled living organisms such as animals and plants, it also plays important roles in the functional
20   co-ordination among family members of single-celled organisms like bacteria. Rapid progress over the last few years has clearly established that N-acyl-homoserine lactones, known as autoinducers

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(AIs), are widely conserved signal molecules in Gram-negative bacteria. AIs were first found in marine bacteria *Vibrio* species in regulation of bioluminescence (Eberhard, *et al.*, 1981; Cao and Meighen, 1989). In recent years, AIs have been identified in a wide range of Gram-negative bacteria. It has been found that AIs are involved in the regulation of a range of biological functions, including Ti plasmid conjugal transfer in *Agrobacterium tumefaciens* (Zhang, *et al.*, 1993), induction of virulence genes in *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Erwinia stewartii*, *Xenorhabdus nematophilus*, *Erwinia chrysanthemi*, *Pseudomonas solanacerum*, and *Xanthomonas campestris* (Jones, *et al.*, 1993; Passador, *et al.*, 1993; Pirhonen, *et al.*, 1993; Pearson, *et al.*, 1994; Beck von Bodman and Farrand, 1995; Barber, *et al.*, 1997; Clough, *et al.*, 1997; Costa and Loper, 1997; Dunphy, *et al.*, 1997; Nasser, *et al.*, 1998), regulation of antibiotics production in *Pseudomonas aureofaciens* and *Erwinia carotovora* (Pierson, *et al.*, 1994; Costa and Loper, 1997), regulation of swarming motility in *Serratia liquifaciens* (Eberl, *et al.*, 1996), and biofilm formation in *Pseudomonas fluorescens* and *P. aeruginosa* (Allison, *et al.*, 1998; Davies, *et al.*, 1998). Many more bacterial species are known to produce AIs but the biological functions related have not been established yet (Bassler, *et al.*, 1997; Dumenyo, *et al.*, 1998; Cha, *et al.*, 1998).

Different bacterial species could produce different AIs. All AI derivatives share identical homoserine lactone moieties but can differ in the length and the structure of their acyl groups. The key

components in AI-mediated gene regulation systems are
LuxI and LuxR type proteins. It has been established
now that LuxI-type protein serves as an autoinducer
synthase that utilizes acyl-ACPs and AdoMet

5   (S-adenosylmethionine) as substrates (More, *et al.*,
1996; Schaefer, *et al.*, 1996). LuxR-type protein is
proposed to be both a receptor for AIs and a
AI-dependent transcriptional regulator that binds DNA
immediately upstream of the *lux* promoter (Meighen,

10   1994; Sitnikov, *et al.*, 1995). A 20-nucleotide
inverted repeat has been identified which is centered
44 nucleotides upstream of the transcription start site
of the luminescence operon. This sequence called *lux*
box is required for transcriptional activation by LuxR

15   and is probably the LuxR binding site (Fuqua, *et al.*,
1994). Similar 18-bp *tra* boxes are found upstream of at
least three TraR-regulated promoters, and disruption of
these elements abolishes transcriptional activation by
TraR (Fuqua and Winans, 1996a).

20   LuxR-type proteins appear to be composed of two
modules (Choi and Greenberg, 1991; Hanzelka and
Greenberg, 1995). Their carboxyl terminal regions
contain a conserved short sequence of 19-amino acid,
putative probe-type helix-turn-helix motif, predicted

25   to be involved in binding to target promoters. A
general mechanism of activation has been proposed by
which the N-terminal domain of LuxR-type protein acts
negatively to prevent an interaction between its
C-terminal domain and the target DNA binding sites.

30   This inhibition can be relieved by the action of an
autoinducer ligand. A strong piece of evidence is that
deletion of the N-terminal domain of LuxR results in
constitutively active alleles of *lux*R, whereas larger

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deletions that remove part of the predicted DNA binding domain abolish transcriptional activation (Choi and Greenberg, 1991). However, other members might use different mechanisms. Recent genetic studies indicate that EsaR and ExpR are likely to be repressors of their target genes rather than activators. Expression of the genes that are repressed by EsaR and ExpR is increased by autoinducers (Beckvon Bodman and Farrand 1995; Throup, *et al.* 1995). It appears that binding of these proteins to their target sites in promoter region causes repression, therefore autoinducer ligands may act to reduce binding affinity.

Evidence that the autoinducer binding site resides in the amino terminal domain of the LuxR protein has been presented (Hanzelka and Greenberg, 1995). LuxR alleles that have mutated amino terminal region require higher level of this signal that does the wild type, indicating this region required for ligand interaction (Slock, *et al.*, 1990; Shadel, *et al.*, 1990). This region (aa 79-127) and a region within the DNA-binding domain (aa 180-230) show a higher degree of conservation among LuxR and its homologs (ca 50% identity) than other parts of these polypeptides. However, the proposed protein-ligand interaction between LuxR and autoinducer has not been proved yet. Analysis of merodiploid *E. coli* strains containing wild-type and mutant LuxR alleles suggested that LuxR functions as a homomultimer and that a region required for multimerization resides within amino acid residues 116 and 161 (Choi and Greenberg, 1992).

BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention relates to an isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein.

In another aspect, the present invention relates to a expression vector which comprises a nucleic acid molecule encoding a bacterial autoinducer inactivation protein, wherein the expression vector propogates in a procaryotic or eucaryotic cell.

In yet another aspect, the present invention relates to a cell of a procaryote or eucaryote transformed or transfected with the expression vector of the present invention.

In yet another aspect, the present invention relates to an isolated protein which has bacterial autoinduction inactivation activity, where the protein comprises the amino acid sequence of SEQ ID NO: 2.

In yet another aspect, the present invention relates to a method for increasing disease resistance in a plant or animal, which method comprises introducing into a cell of such plant or animal a nucleic acid sequence which encodes a bacterial autoinducer inactivation protein in a manner which allows said cell to express said nucleic acid sequence.

In yet another aspect, the present invention relates to a method of preventing or reducing bacterial damage to a plant or animal, which method comprises administering to a plant or animal in need of such prevention or reduction an effective amount of a bacterial autoinducer inactivation protein.

In yet another aspect, the present invention relates to a composition for reducing bacterial damage to a plant or animal, which comprises:

a) an effective amount of a bacterial autoinducer
inactivation protein; and
b) a suitable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

5   Figure 1 shows the time course of AIs inactivation
by cell extracts from Bacillus sp. strain 240BI. Cell
extracts in 0.2 M phosphate buffer (pH 7.0) containing
100 ug total protein were added to the same buffer
containing OHHL in a final concentration of 20 uM. The
10   reaction was conducted in a 1.5 ml Eppendorf centrifuge
tube in a final volume of 200 microliters and incubated
at 28°C. Same concentration of OHHL in the phosphate
buffer was used as control. Samples were taken at
10-min interval till 60 min and the reaction was
15   stopped by boiling for 3 min. The samples were
centrifuged for 5 min in a bench top centrifuge at the
top speed and then assayed for AIs activity as
described (Zhang, 1993). Blue colony indicates the
presence of AI that activates the lacZ reporter gene,
20   and white colony indicates absence of AI. Rows from
left to right: 1, OHHL control without protein extract;
2 - 7, samples after 10, 20, 30, 40, 50, 60 min enzyme
reaction.

Figure 2 shows the estimation of molecular mass of
25   AIs inactivation enzyme. A 600 µl aliquot of cell
extracts was added to the Centricon 30 (Amicon) and was
centrifuged at a speed of 5000 x g for 30 min at 4°C.
Passing fraction (550 microliters) and un-passing
fraction (50 microliters) were topped up separately to
30   a final volume of 600 microliters by adding 0.2 M
phosphate buffer (pH 7.0). For bioassay, different

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amounts of protein samples were added to the tubes containing OHHL in a final concentration of 20 ,uM. From row 1 to 6, protein samples added were 2, 4, 6, 8, 10 and 0 ,ul and the final reaction volume was 20

5   microliters for each reaction. Plate A: Passing fraction, Plate B: un-passing fraction.

Figure 3 shows the cloning and deletion analysis of Bacillus SP. strain 240B1 AI inactivation region. Cosmid clone E7-R3 contains the 4.3-kb EcoRI fragment

10   identified by restriction analysis of overlapping cosmid clones. For deletion analysis, the same fragment was cloned into cloning vector pGEM-7Zf(+) for generation of clone E7-7. The deletion subclones were produced by restriction enzyme digestion and Dnase I

15   treatment from the clone E7-7. The location and direction of Ptac promoters in the cosmid and in the pGEM-7Zf(+) clone are indicated by arrows. AI inactivation activity of the clones is shown in the second column: +, with AI inactivation activity; -,

20   without Al inactivation activity. Restriction enzymes: E, EcoRI; H, HindIII; Ev, EcoRV; St, StyI. The location and direction of transcription of the aiiA ORF is indicated by an open arrow.

Figure 4A shows the nucleotide sequence of the

25   aiiA gene [SEQ ID NO:1]. The potential ribosome binding sequence and -10 promoter element are underlined and double underlined respectively. The coding portion starts at base 1. The putative factor-independent termination site is labeled by a

30   thick underline. Figure 4B shows the predicted amino acid sequence of the aiiA gene product [SEQ ID NO:2].

A short peptide sequence similar to the aspartyl
protease active site consensus motif is underlined.

Figure 5 shows the best match of amino acids
sequence of *aiiA* gene product (AiiA) to the concensus
aspartyl proteases active site motif (Asp). Symbol: X,
any amino acid. A vertical line indicates perfect
match.

Figure 6 shows the bioassay for AIs inactivation
activities in *Bacillus sp.* strain 240B1, E.coli clones
and AIs production activity in *Erwinia carotovora*
strains. Row 1, OHHL control; row 2, *Bacillus* sp.
strain 240BI; row 3, *E. coli* DH5α; row 4, *E. coli* DH5α
(pE7-R3); row 5, *E. coli* DH5α (pF41); row 6, *Erw.
carotovora* SCG1(pE7R3); row 7, *Erw. carotovora*
SCG1(pLAFR3); row 8, *Erw. carotovora* SCG1. In the
bioassay, OHHL was added to a final concentration of 20
μM to the samples from lines 1 to 5. No exogenous AIs
were added to the samples from rows 6 to 8.

Figure 7 shows the effect of *aiiA* gene expression
in *Erw. carotovora* on pathogenicity in (A), potato;
(B), eggplant; (C), Chinese cabbage; (D), carrot; and
(E), celery. Top: plant tissues were inoculated with
Erw. carotovora SCG1. Bottom: plant tissues were
inoculated with *Erw. carotovora* SCG1 (pE7-R3). The
actively growing bacteria were centrifuged for 1 min at
3000 x g, resuspended with YEB liquid medium to OD600 =
1.3 (2 x 10^9 cfu/ml) which was designed as 10^0 inoculum.
The 10^0 inoculum was diluted 5 and 10 times respectively
to prepare $10^{-1/2}$ and 10^{-1} dilutions. The Plant tissues
were inoculated by adding a 4-μl volume of bacteria

inoculum to the freshly cut surface or a wounding site punched by a pipette tip. The inoculum concentration from the left to the right plate: 10^0; $10^{-1/2}$; and 10^{-1}. The inoculated plant tissues were placed in plastic plates and incubated at 28°C. The photograph was taken 48 h after inoculation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that the SEQ ID NO:2 protein has the effect of reducing or eliminating the activity of bacterial autoinducers (AIs). Consequently, the protein, and any nucleic acid that encodes the protein, may be used in a variety of situations where it is desired to reduce or eliminate the effect of such bacteria.

In one preferred aspect, the present invention provides a nucleic acid molecule which is selected from the group consisting of:

a) a nucleic acid having the sequence of SEQ ID NO:1;

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:2; and

c) a nucleic acid that hybridizes to a) or b) above, wherein a positive hybridization signal is observed after washing with 1 X SSC and 0.1% SDS at 55°C for one hour. The nucleic acid optionally further comprises a signal peptide coding region of any sequence.

The nucleic acid sequence may be used to confer bacterial resistance in plants or animals. A nucleic acid that encodes a bacterial autoinducer inactivation protein can be introduced into a cell such that the

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inactivation protein is expressed by the plant or animal.

The nucleic acid sequence may be used to confer resistance to diseases where the expression of pathogenic genes are regulated by autoinducers, such as the diseases caused by *Pseudomonas aeruginosa*, *Erwinia stewartii*, *Xenorhabdus nematophilus*, *Erwinia chrysanthemi*, *Pseudomonas solanacerum*, and *Xanthomonas campestris* (Passador, *et al.*, 1993; Pirhonen, *et al.*, 1993; Pearson, *et al.*, 1994; Beck von Bodman and Farrand, 1995; Barber, *et al.*, 1997; Clough, *et al.*, 1997; Costa and Loper, 1997; Dunphy, *et al.*, 1997; Nasser, *et al.*, 1998). Preferably, in the agricultural setting, the sequence may be used to confer soft rot disease resistance in susceptible plants, such as potato, eggplant, Chinese cabbage, carrot and celery.

The sequence may be introduced into plant or animal cells by well-known methods. Methods for the transformation or transfection of eukaryotic cells with exogenous nucleic acid sequences include transfection, projectile bombardment, electroporation or infection by *Agrobacterium tumefaciens*. These methods are likewise familiar to the person skilled in the area of molecular biology and biotechnology and need not be explained here in detail. As pathogenic bacteria cells are confined to the intercellular area of plant tissues, it is desirable to target the AiiA protein into the intercellular spaces. Such may be accomplished by fusing a secretion signal peptide to the AiiA protein (Sato, *et al.*, 1995; Firek, *et al.*, 1993; Conrad and Fiedler, 1998; Borisjuk, *et al.*, 1999). Alternatively, a plant membrane attachment motif can be incorporated into the peptide sequence of AiiA for anchoring the

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AiiA enzyme in the outer surface of plant cell membrane.

The present invention provides a new strategy for engineering resistance to diseases. In particular, this strategy targets N-acyl homoserine lactone autoinducers that induce expression of pathogenic genes of many bacterial pathogens at a threshold concentration. This strategy is applicable to all plant, animal or mammal diseases where the expression of pathogenic genes of the bacterial pathogens is inducible by N-acyl homoserine lactone autoinducers.

The present invention also contemplates usage of a bacterial autoinducer inactivation protein directly to treat or prevent bacterial damage. For example, the protein may be applied directly to plants in need of such treatment or prevention. In a preferred embodiment, the protein is applied in the form of a composition which comprises an effective amount of the protein and a suitable carrier. The composition may have a wide variety of forms, including solutions, powders, emulsions, dispersions, pastes, aerosols, etc.

The bacterial autoinducer inactivation protein may also be used to treat bacterial infections in animals, including humans. In that application, an effective amount of the active ingredient is administered to an animal in need of such treatment.

For therapeutic treatment, the active ingredient may be formulated into a pharmaceutical composition, which may include, in addition to an effective amount of the active ingredient, pharmaceutically acceptable carriers, diluents, buffers, preservatives, surface active agents, and the like. Compositions may also include one or more other active ingredients if necessary or desirable.

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The pharmaceutical compositions of the present invention may be administered in a number of ways as will be apparent to one of ordinary skill in the art. Administration may be done topically, orally, by inhalation, or parenterally, for example.

Topical formulations may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Oral formulations include powders, granules, suspensions or solution in water or non-aqueous media, capsules or tablets, for example. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be used as needed.

Parenteral formulations may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The dose regimen will depend on a number of factors which may readily be determined, such as severity and responsiveness of the condition to be treated.

Aspects of the invention will now be illustrated with reference to the following non-limiting examples.

EXAMPLE 1

Bacterial isolate 240B1 was isolated from soil suspension based on its function for inactivation of N-ß-oxo-hexanoyl-L-homoserine lactone (OHHL) and N-ß-oxooctanoyl-L-homoserine lactone (OOHL) and N-ß-oxodecanoyl-L-homoserine lactone (ODHL) (Zhang, *et al.*, 1993). Unless otherwise stated, OHHL was used for routine bioassay. *Erwinia carotovora* strain SCG1 was isolated from Chinese cabbage leaf showing soft rot symptoms. It has been confirmed that strain SCG1 produces AIs and elicits soft rot disease in potato and

Chinese cabbage. *Escherichia coil* strain DH5α was used as a host for DNA cloning and subcloning. *Agrobacterium tumefaciens* strain NT1 (*traR; tra::lacZ749*) was used as an indicator in bioassay for

5   AI activity (Piper, *et al.*, 1993). *E. coli* strain was cultured in Luria-Bertani (LB) medium at 37°C and other strains were cultured in LB (Miller, 1972) or YEB medium (per liter contains: casein hydrolysate 10 g, yeast extract 5 g, NaCl 10 g, sucrose 5 g, MgSO$_4$·7H$_2$O 0.5

10 g, agar 15 g, pH 7.2) at 28 °C. The minimal salts medium with mannitol and (NH4)$_2$SO$_4$ as carbon and nitrogen sources was used for bioassay of OHHL (Petit and Tempe, 1978). Appropriate antibiotics were added as indicated at the following concentrations:

15 ampicillin, 100 µg/ml; tetracycline, 20 µg/ml and kanamycin, 50 µg/ml.

Bioassay of AIs activity

The qualitative and quantitative bioassay methods for determination of AIs activity has been described

20 previously (Zhang, 1993). For determination of the AIs production ability of wild-type and genetically modified *Erwinia* strains, the same bioassay procedure was used except that no OHHL was added into the bacterial culture.

25 ### Cloning and sequencing of the AiiA gene

Genomic DNA from 240B1 was digested partially with *Eco*RI. DNA fragments were ligated to the dephosphorylated *Eco*RI site of cosmid vector pLAFR3 (Staskawicz, *et al.*, 1987). Ligated DNA was packaged

30 with Gigapack III XL Packaging Extract (Stratagene) and transfected into *E. coil* DH5∝. Cosmid clones with OHHL

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inactivation activity were identified by using the
bioassay method described above. Subcloning into
sequencing vector pGEM-7Zf(+) was carried out by
routine techniques (Sambrook, *et al.*, 1989). Deletion

5   analysis was carried out by using DnaseI method as
described by Lin, *et al.* (1985). The sequencing was
performed on both strands using the ABI PRISM™
dRhodamine Terminator Cycle Sequencing Ready Reaction
Kit (PE Applied Biosystems). Nucleic acid sequence

10   data and deduced amino acid sequences were analyzed
with a DNASTAR™ sequence analysis software package
(DNASTAR Inc.) and database searches were performed
using the BLASTA search algorithm (Altschul, *et al.*,
1990).

15   ***Genetic modification of Erwinia strain SCG1***

The E7-R3 plasmid, carrying the *aiiA* gene in the
cosmid vector pLAFR3, was transferred into *Erwinia*
stain SCG1 by triparental mating with the helper strain
RK2013 (Ditta, *et al.*, 1980). Transconjugants were

20   selected on the plates containing minimal medium with
tetracycline and confirmed by PCR with primers specific
to the *aiiA* gene.

Virulence tests

The virulence of wild-type *Erw. carolovora* strain

25   SCG 1 and the aiiA gene transformant SCG1(E7-R3) was
evaluated by inoculation. Four µl of early stationary
phase bacterial suspension (containing ~2 x 10^9 cell/ml)
or diluted bacteria was added to the cut surfaces or
wounding sites of plant tissues. The inoculated plant

30   tissues were incubated in a Petri dish at 28°C

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overnight. The severity of soft rot was examined 48
hours after incubation.

Results
Screening of bacteria that inactivate AIs

5   Bacterial isolates from plant and soil samples
were screened for enzymatic inactivation of AIs. A
bacterial isolate 240B1, which showed a strong ability
to eliminate AIs activity, was selected for further
study. The total protein extracts from isolate 240B1
10   eliminated AIs activity completely during one-hour
incubation (Fig. 1), and the capacity of the protein
extract to inactivate AIs was abolished by treatment
with proteinase K for 1 hour or boiling for 5 min.
These observations indicate enzymatic inactivation of
15   AIs by bacterial isolate 240B1. The isolate was
taxonomically characterized as *Bacillus* sp., because of
the following characteristics: Gram-positive,
rod-shaped, catalase positive, facultatively anaerobic,
and 16 rRNA sequence homology to that of other *Bacillus*
20   bacteria (data not shown).

The molecular mass of the enzyme for AIs
inactivation appears to be larger than 30 kDa. Its
activity was lost after passing the protein extract
through Centricon 30 (Amicon) but the activity was
25   recovered in the re-suspended fraction that failed to
pass the Centricon 30 (Fig. 2).

Cloning and localization of AIs inactivation region

To identify the gene encoding AIs inactivation, a
cosmid library was constructed with the genomic DNA of
30   *Listera* sp. strain 240B1. Twelve hundred clones were
screened for AIs inactivation activity. Three clones

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showing AIs inactivating function were identified. Restriction analysis showed that the 3 clones shared one common band of 4.3-kb generated by EcoRI digestion. The bioassay with the subclone E7-7 containing this

5   4.3-kb EcoRI fragment confirmed that this fragment encodes AIs inactivation function (Fig. 3). To identify the minimum size and the location of the AIs inactivation gene (aiiA), a serial of deletion clones was generated by deletion from both ends of this 4.3-kb

10   fragment with the DNaseI method (Lin, et al., 1985). The results indicated that the aiiA gene is contained in a 1.2 Kb fragment in clone F41 (Fig. 3).

AiiA gene encodes a novel protein

The 1.2-kb DNA insert in clone F41 was completely

15   sequenced from both strands. The nucleotide sequence of aiiA and the predicted amino acid sequence are shown in Fig. 4. The complete sequence of the DNA insert contains 1,222 base pairs and there are 4 potential in-frame open reading frames (ORF) starting from

20   nucleotide position of 1, 42, 156 and 228 respectively (Fig. 4). Deletion analysis indicated that only the longest ORF encodes AIs inactivation function, because the clone R34, in which the 48 bp promoter region and nucleotides from 1 to 13 in the longest ORF were

25   deleted, lost AI inactivation function completely, although the remaining DNA insert was placed under the control of a functional Ptac promoter (Fig. 3). This is confirmed by fusing the longest ORF to the glutathione S-transferase gene in the same ORF and

30   testing for AI inactivation activity of the purified fusion protein (data not shown). This ORF contains 750 bp nucleotide and encodes a protein of 250 amino acids,

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with a predicted molecular mass of 28,036 daltons and
an isoelectric point at 4.7, because of 19 strongly
basic and 39 strongly acidic amino acids residues. The
putative initiation codon is preceded at a spacing of 7
bp by a potential ribosome-binding sequence (AAGGTGG)
which is complementary to the 3' end of the *E. coli* 16S
rRNA. The best sequence match (TATTGT) to the
consensus -10 promoter element (TATAAT) occurs 35 bp
upstream of the initiation codon. A TCTT box following
a T-rich region resembling the potential factor-
independent termination site is found downstream of the
termination codon (Brendel, 1986). The total GC
content of the *aiiA* gene is 37% and GC content in the
third position of the codon is 27.2%.

Database searches showed that the aiiA gene has no
significant similarity to known sequences in the major
databases (GenBank, European Molecular Biology
Laboratory, Protein Information Resource, and
Swiss-Prot) by FASTA and BLAST analysis at either
nucleotide or peptide sequence level, suggesting that
AiiA is a novel protein. Consensus protein motif
search using the Genetics Computer Group (Madison, WI)
MOTIF program showed that a short peptide sequence,
"ILVDTGMPESAV" from position 47 to 58 in AiiA, is
similar but not identical to the aspartyl protease
active site signature pattern (Rawlings and Barrett,
1995) (Fig. 5).

**Expression of aiiA gene in Erwinia carotovora decreases
AIs releasing and attenuates virulence**
The cosmid clone E7-R3 was transferred into
Erwinia carotovora strain SCG1 by triparental mating.
The pLAFR3 vector has been safely maintained in *Erwinia*

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carotovora without selection pressure. The bioassay showed that the AIs released by *Erwinia carotovora* (E7-R3) was significantly reduced (Fig. 6, lane 6), while the presence of the cosmid vector pLAFR3 alone in *Erwinia carotovora* did not affect AIs production (Fig. 6, lanes 7). Data suggest that the most of AIs produced by *Erwinia carotovora* strain SCG1 was inactivated by *aiiA* gene product.

The *Erwinia carotovora* SCG1(E7-R3) that expresses AiiA protein failed to or caused only minor soft rot disease symptom in potato, eggplant, Chinese cabbage, carrot and celery, while its parental strain caused severe symptoms (Fig. 7A, B, C, D, E). To prevent experimental errors due to genetic variations, four colonies from *Erwinia carotovora* strain SCG1 and its *aiiA* gene transformants respectively, were randomly selected for testing AIs production and virulence on potato. Similar results were obtained in both experiments. The *Erwinia carotovora* strain SCGI (pLAFR3) that contains the cosmid vector only caused the same level of disease severity as its parental strain *Erwinia carotovora* strain SCGI (Fig. 7F).

Discussion

Bacterial isolate 240B I, which was identified as *Bacillus* sp., produces an enzyme that can effectively inactivate the three AIs tested, *i.e.*, N-ß-oxo-hexanoyl-L-homoserine lactone, N-ß-oxo-octanoyl-L-homoserine lactone and N-ß-oxo-decanoyl-L-homoserine lactone. The gene (*aiiA*) encoding the AI inactivation enzyme has been cloned and fully sequenced. Expression of the *aiiA* gene in transformed *E. coli* and pathogenic bacteria *Erwinia carolovora* confers ability for AI

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inactivation and significantly reduces the AIs release from *Erwinia carolovora*. To our knowledge, it is the first protein identified capable of enzymatic inactivation of N-acyl-homoserine lactones, the

5 autoinducers for global gene regulation in a diverse of bacteria species.

The AiiA is a novel protein. There is no significant homology to known proteins in major databases. It shares similarities to the consensus

10 pattern of the aspartyl proteases active site (Rawlings and Barret, 1995). Aspartyl proteases, also known as acid proteases, are widely distributed in vertebrates, fungi, plants, retroviruses and some plant viruses. The aspartyl proteases from most retroviruses and some

15 plant viruses are homodimers. The molecular mass of AiiA protein is about 28 kDa but it failed to pass a molecular sieve with a cut off size of 30 kDa, indicating a possibility that AiiA protein exists as a homodimer or homomultimer under the natural conditions.

20 However, there is also a possibility that AiiA monomer has an irregular three-dimensional structure, which hinders it passing through the molecular sieve. Aspartyl proteases are endopeptidases and hydrolyses amide linkages of proteins. Crystallographic study has

25 shown that the enzyme of the aspartyl protease family are bilobed molecules with the active-site cleft located between the lobes, and each lobe contributing one of the pair of aspartic acid residues that is responsible for the catalytic activity (Sielecki et

30 al., 1991).

Erwinia carotovora is a plant pathogen that produces and secretes exoenzymes that act as virulence determinants for soft rot diseases of various plants

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including potato, cabbages, tomato, chili, carrot, celery, onion, and lettuce (Kotoujansky, 1987).

Mutants that were defective in the producing *N*-3-(oxohexanoyl)-L-homoserine lactone were also defective in systhesis of the pectinase, cellulase and protease exoemzymes. These mutants failed to induce soft rot disease in potato tubers (Jones, *et al.*, 1993). It was found that the *expI* gene, which is homologous to *luxI* gene of *Vibrio fischeri*, encodes autoinducer production in *Erwinia carotovora*. The *expI* mutant was avirulent when it was inoculated to tobacco leaf but the virulence was restored by external antoinducer addition (Pirhonen, *et al.*, 1993). Obviously, autoinducers are a potential target for genetic engineering of plant soft rot disease resistance. As an interim test and a concept proving approach, the cosmid clone containing the *aiiA* gene was introduced to *Erwinia carotovora* strain SCG1. Expression of the AiiA enzyme in *Erwinia carotovora* significantly reduced the release of autoinducers, and the genetically modified *Erwinia carotovora* that expressed AiiA failed to induce any or induce only minor soft rot disease symptom on all plants tested, including potato, eggplant, Chinese cabbage, carrot and celery. Our results further support the important role of autoinducers in the regulation of expression of virulence genes in *Erwinia carotovora*, and the potential of the *aiiA* gene to confer resistance to soft rot disease and other diseases in which the autoinducers are involved in regulation of pathogenic gene expression.

The present invention provides a new strategy for engineering resistance to diseases. In particular,

this strategy targets N-acyl homoserine lactone
autoinducers that induce expression of pathogenic genes
of many bacterial pathogens at a threshold
concentration. By using the above-memtioned

5 conception-proving approach, the present invention
demonstrates that reduction or elimination of
autoinducers produced by pathogenic bacteria by an
autoinducer inactivation enzyme significantly
attenuates pathogenicity of otherwise virulent

10 bacterial pathogen. Because the expression of
pathogenic genes in pathogenic bacteria requires a
threshold concentration, this AI-inactivation strategy
is applicable to all plant, animal or mammal diseases
where the expression of pathogenic genes of the

15 bacterial pathogens is inducible by N-acyl homoserine
lactone autoinducers.

The *aiiA* gene could also be a useful tool for
investigation of the role of AIs in those bacteria
where the biological functions regulated by AIs has not

20 been established. In recent years, many more bacteria
species have been shown to produce AIs (Bassler, *et
al.*, 1997; Dumenyo, *et al.*, 1998; Cha, *et al.*, 1998;
Surette, *et al.*, 1999). Some of them are important
plant pathogens such as *Psendomonas* and *Xanthomonas*

25 species. The gene knock out approach based on sequence
homology could be difficult. The overall levels of
sequence similarity of AIs synthase and the related
regulatory protein from different genera are rather
low, often no higher than 28-35% identity between LuxI-

30 type proteins and 18-25% identity for LuxR-type
proteins (Fuqua et al., 1996). However, it is feasible
and simple to introduce the *aiiA* gene into these

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bacteria to probe the biological functions regulated by AIs.

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5

CLAIMS

1. An isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein.

2. The molecule of claim 1, wherein the nucleic acid molecule is selected from the group consisting of:

a) a nucleic acid having the sequence of the coding portion of SEQ ID NO:1;

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:2; and

c) a nucleic acid that hybridizes to a) or b) above, wherein a positive hybridization signal is observed after washing with 1 X SSC and 0.1% SDS at 55°C for one hour.

3. The molecule of claim 1, which further comprises a signal peptide coding region of any sequence.

4. An expression vector which comprises the nucleic acid molecule of claim 1, wherein the expression vector propogates in a procaryotic or eucaryotic cell.

5. A cell of a procaryote or eucaryote transformed or transfected with the expression vector of claim 4.

6. An isolated protein which has bacterial autoinduction inactivation activity, where the protein comprises the amino acid sequence of SEQ ID NO: 2.

7. A method for increasing disease resistance in a plant or animal, which method comprises introducing

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into a cell of such plant or animal a nucleic acid sequence which encodes a bacterial autoinducer inactivation protein in a manner which allows said cell to express said nucleic acid sequence.

5   8.   The method of claim 7, wherein the nucleic acid sequence is selected from the group consisting of:

a)   a nucleic acid having the sequence of the coding portion of SEQ ID NO:1;

b)   a nucleic acid encoding the amino acid
10   sequence of SEQ ID NO:2.

9.   The method of claim 7 or 8, wherein the nucleic acid sequence further comprises a signal peptide coding region of any sequence.

10.   The method of claim 7 or 8, wherein the
15   nucleic acid sequence further comprises a membrane attachment domain-coding region of any source.

11.   The method of claim 7, wherein the plant is susceptible to bacterial soft rot disease.

12.   The method of claim 11, wherein the plant is
20   selected from the group consisting of potato, eggplant, Chinese cabbage, carrot and celery.

13.   The method of claim 7, wherein the plant is susceptible to a bacterial disease in which the expression of a virulence gene is regulated by an N-
25   acyl homoserine lactone autoinducer.

14.   A method of preventing or reducing bacterial damage to a plant or animal, which method comprises

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administering to a plant or animal in need of such prevention or reduction an effective amount of a bacterial autoinducer inactivation protein.

15. The method of claim 14, wherein the protein comprises SEQ ID NO: 2.

16. A composition for preventing or reducing bacterial damage to a plant or animal, which comprises:

a) an effective amount of a bacterial autoinducer inactivation protein; and

b) a suitable carrier.

17. The composition of claim 16, wherein the protein comprises SEQ ID NO: 2.

18. A method for screening of bacterial isolates for autoinducer inactivation activity, which comprises:

a) isolating a single colony bacterial culture from soil or plant samples;

b) screening the culture for autoinducer inactivation activity;

c) preparing a crude protein extract from the culture; and

d) confirming enzymatic inactivation of autoinducer activity by the crude protein extract.

19. A method of isolating the nucleic acid of claim 1 or claim 2, which comprises the steps of:

a) preparing a gene bank from a donor organism that contains a nucleic acid sequence coding for a protein with an autoinducer inactivation activity in a suitable host organism;

b) screening the clones of the gene bank; and

c) isolating the clones which contain a nucleic acid coding for a protein with autoinducer inactivation activity.

20. A process as claimed in claim 19, wherein *E. coli* is used as host organism.

21. A process as claimed in claim 19, wherein the steps of preparing a gene bank, screening the clones, and isolating the clones are performed in an *E. coli* strain that does not inactivate the autoinducer.

22. A method which comprises:
    a) introducing the nucleic acid sequence of claim 1 or claim 2 into a bacterial cell; and
    b) screening the bacterial cell obtained from step a) for changed biological function.

23. The method of claim 22, wherein the changed biological function is a function which is lost as a result of step a).

24. The method of claim 22, wherein the changed biological function is a function which is suppressed as a result of step a).

25. The method of claim 22, wherein the changed biological function is a function which is enhanced as a result of step a).

ABSTRACT OF THE DISCLOSURE

Disclosed are an isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein, the encoded protein, and anti-bacterial uses therefor.
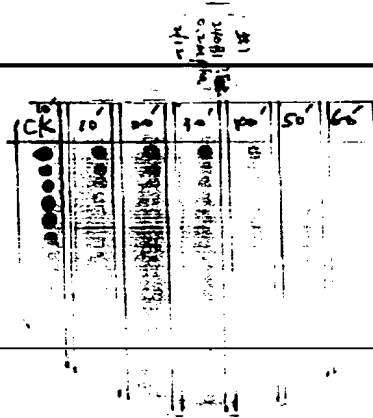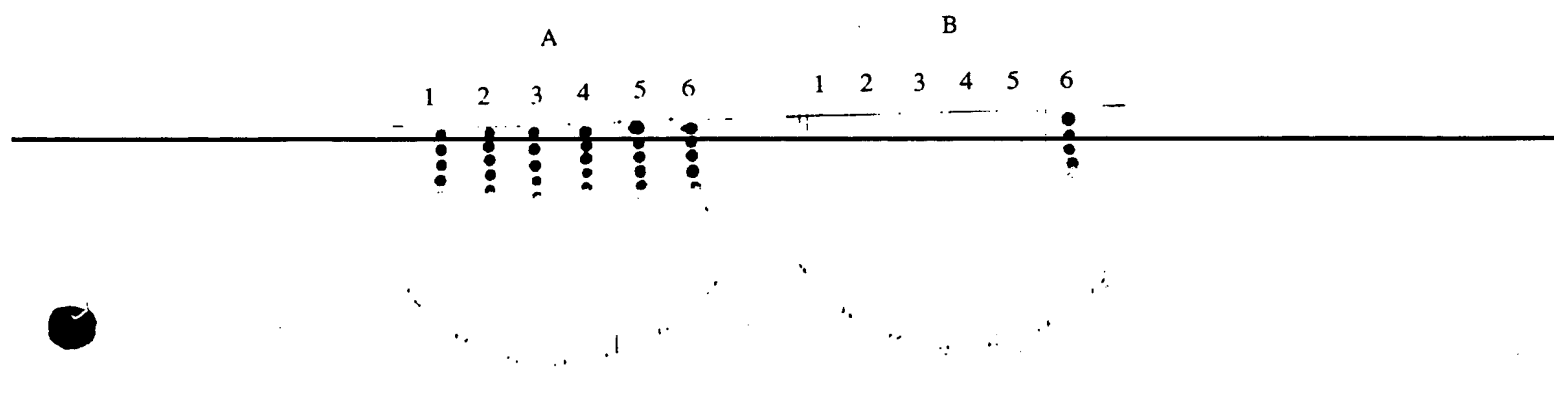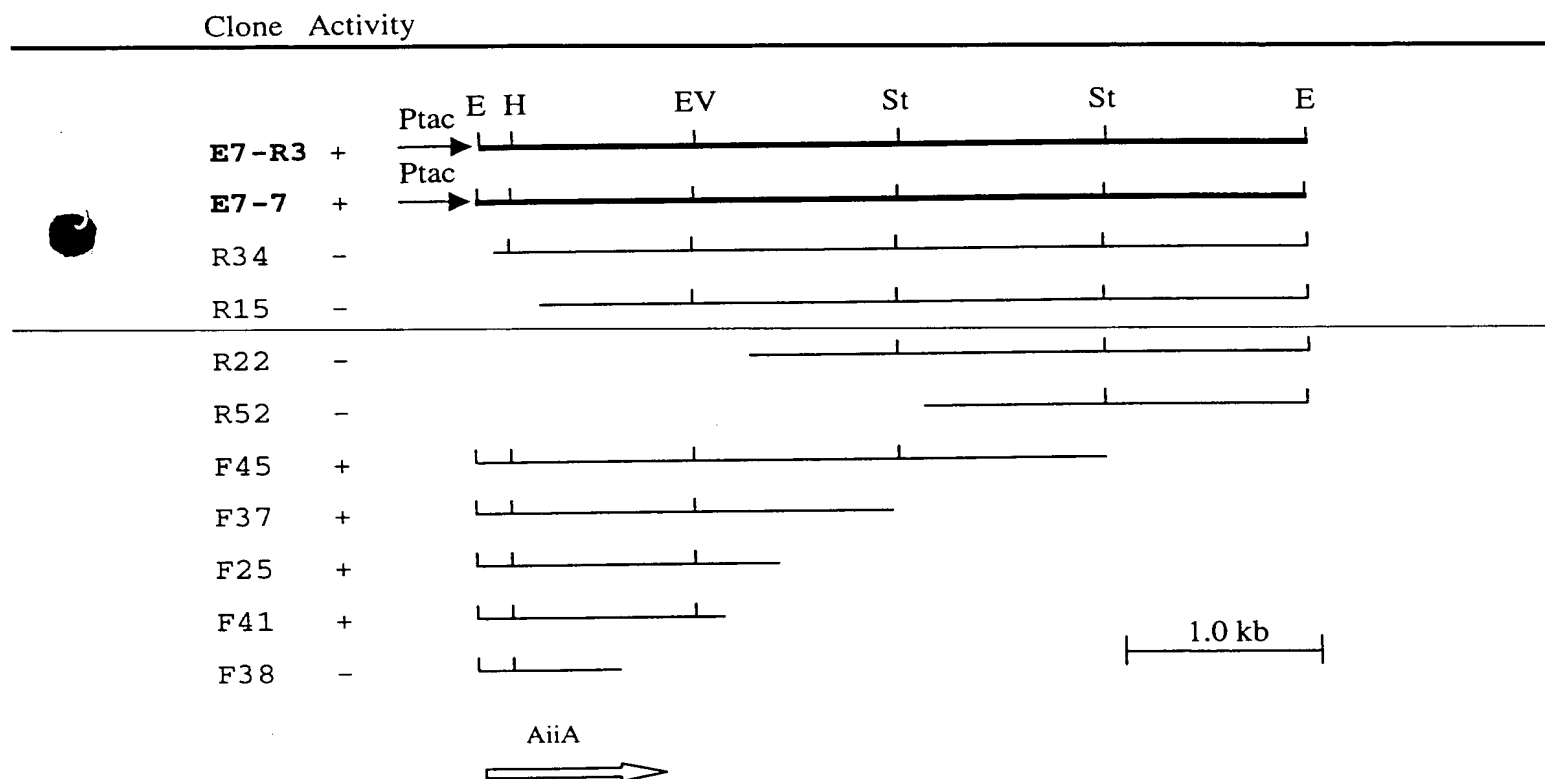
Figure 1

Figure 2

25

Figure 3

Figure 4(A)

..ctttactg<u>tattgt</u>ttttattcaaaactaaatgta<u>aaggtgg</u>atacata -1

ATGACAGTAAAGAAGCTTTATTTCGTCCCAGCAGGTCGTTGTATGTTGGA 50

TCATTCGTCTGTTAATAGTACATTAACACCAGGAGAATTATTAGACTTAC 100

CGGTTTGGTGTTATCTTTTGGAGACTGAAGAAGGACCTATTTTAGTAGAT 150

ACAGGTATGCCAGAAAGTGCAGTTAATAATGAAGGTCTTTTTAACGGTAC 200

ATTTGTCGAAGGGCAGGTTTTACCGAAAATGACTGAAGAAGATAGAATCG 250

TGAATATTTTAAAACGGGTTGGTTATGAGCCGGAAGACCTTCTTTATATT 300

ATTAGTTCTCACTTGCATTTTGATCATGCAGGAGGAAATGGCGCTTTTAT 350

AAATACACCAATCATTGTACAGCGTGCTGAATATGAGGCGGCGCAGCATA 450

GCGAAGAATATTTGAAAGAATGTATATTGCCGAATTTAAACTACAAAATC 500

ATTGAAGGTGATTATGAAGTCGTACCAGGAGTTCAATTATTGCATACACC 550

AGGCCATACTCCAGGGCATCAATCGCTATTAATTGAGACAGAAAAATCCG 600

GTCCTGTATTATTAACGATTGATGCATCGTATACGAAAGAGAATTTTGAA 650

AATGAAGTGCCATTTGCGGGATTTGATTCAGAATTAGCTTTATCTTCAAT 700

TAAACGTTTAAAAGAAGTGGTGATGAAAGAGAAGCCGATTGTTTTCTTTG 750

GACATGATATAGAGCAGGAAAGGGGATGTAAAGTGTTCCCTGAATATATA 800

TAGtacaaaaagtcatgagcttattcgctcatgacttttttcgtttaaatg 850

Atttttttaaataagttataaactttttttagaacta<u>tctt</u>catttaattg 900

Atagtacgtaaggtttacatcattaggagtatcttgttgagcaatcatca 950

Cttcgttactgtgatggtcaactacccatatgaaatattttttataagtc 1000

Ccatcctcgaaagtaatccacatatcacagtctattaaatctgatccttc 1050

Ttcatctaatgttaattttccttttttggcggtatccatactgttaatga 1100

Atgttttttaattcatctgttttttgtgagaaagatatcctttttttgttttta 1150

28

Attgactcgacatgtatatctttatttcttgttttcctaaaaagacagg 1200

gggctcatttgggtctctttgagt 1222

Figure 4(B)

MTVKKLYFVPAGRCMLDHSSVNSTLTPGELLDLPVWCYLLETEEGPILVD 50

TGMPESAVNNEGLFNGTFVEGQVLPKMTEEDRIVNILKRVGYEPEDLLYI 100

ISSHLHFDHAGGNGAFINTPIIVQRAEYEAAQHSEEYLKECILPNLNYKI 150

IEGDYEVVPGVQLLHTPGHTPGHQSLLIETEKSGPVLLTIDASYTKENFE 200

NEVPFAGFDSELALSSIKRLKEVVMKEKPIVFFGHDIEQERGCKVFPEYI 250

Figure 5

Asp: [LIVMFGAC]-[LIVMTADN]-[LIVESA]-D-[ST]-G-[STAV]-
          | | | | | |
AiiA: I₄₇   L   V   D   T G   M

Asp: [STAPDENQ]-X-[LIVMFSTNC]-X-[LIVMFGTA]
            | | | |
AiiA: P   E   S   A   V₅₈

30

Figure 6

Figure 7